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Effect the alteration of pH has upon a basic and acid dye for use in a modified bacterial capsule staining technic

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THROUGH THE ALTERATION OF pH AND [illegible] A [illegible]
ACID DYE FOR USE IN A [illegible] BACTERIAL
CAPSULE STAINING TECHNIC

A thesis

by

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INTRODUCTION

[illegible] I

Most observers contend that the majority of bacteria possess a capsule. However, this extracellular envelope is ill-defined and difficult to demonstrate in many species. The bacterial capsule has been correlated with the virulence of many organisms. This correlation brought the advent of much research, around 1900, pertaining particularly to the physico-chemical makeup of the capsule. There were many staining methods devised at this time to demonstrate the capsule in different species. The organism of primary concern in these staining procedures was Diplococcus pneumoniae.

Wadsworth recorded his experience with old and new capsular stains up to 1906 (21). He found that the methods of Friedlander, Johne, Muir, MacConkey, [illegible], and [illegible] and [illegible] for staining capsules with aqueous solutions of gentian violet or carbol fuchsin were unsatisfactory in that the preparations often varied. The methods of [illegible], Welch, and [illegible] were more reliable though complicated. A review of all the new capsule staining methods shows them to include [illegible] involving the use of numerous chemicals and fixatives.

Hiss published a [illegible] devised in 1905 ( ). [illegible] and is now used extensively as a routine method. The Hiss procedure is comparatively simple but a certain amount of experience and judgment is needed if a satisfactory stain is to be secured.

Another staining method was used by Buerger to note the morphology of the capsule with reference to *Diplococcus pneumoniae* and *Streptococcus* (4). His technic required the use of various chemicals and mordants. Although the method was complicated, it was reliable.

Many methods for staining the bacterial capsule were found to be satisfactory by various observers. However, the majority of these stains were very intricate. The purpose of this study was to note if a basic or acid dye altered to a certain pH could be used in a practical, simplified, and reliable capsule staining procedure.

A definite step in the introduction of a simple capsule staining method was made by Muntoon (12). The staining material comprised many chemicals, but the procedure took only 1 minute to complete. The fixing and staining material used in this method was made up of 2% carbolic acid 1 cc., concentrated lactic acid 0.25-0.5cc. 1% acetic acid 1 cc. and saturated alcohol solution of basic fuchsin 1 cc., carbol fuchsin (old) 1 cc. The acidic solutions used in this method suggest that the mechanism involves simply the lowering of the pH of the solution in question.

A capsule staining method involving basic fuchsin as the stain, and methylene blue in the counter stain was reported by Leifson (13). The capsule was stained red and the organism was stained blue. Gins' method (6) using India ink, demonstrated the capsule in relief. Still another technic, devised by Churchman (5) employing Wright's stain was used with fair results. Anthony (1) reported one of the latest capsule stains in 1931. His procedure was very simple. He used 1% aqueous solution of crystal violet as the stain and washed with 20% copper sulfate.

General nature of the capsule

The capsule is a viscous material which remains concentrated around many bacterial species. The ability to produce the capsular material is a hereditary characteristic which is lost when the culture undergoes phase variation or dissociation.

Little is known concerning the influence of environment on capsule production. There are, however, some environmental factors which influence the size and formation of the capsule. It has been shown that cultures of the colon-typhoid group grown at low temperatures (18-20° C.) in presence of fermentable carbohydrates capsules are produced (15). [illegible] (11) found that the largest capsules produced by *Klebsiella* pneumoniae were obtained by cultivation for about 6 hours at 37° C. in a medium containing 1-½% peptone and 1% glucose. It was demonstrated also that encapsulation, for this species occurs under conditions unfavorable for growth and not when active proliferation occurs. Both encapsulated organisms produce large capsules in young cultures. Young organisms of Streptococcus hemolyticus demonstrated large capsules, but these capsules degenerated in old ones (17). Morison (16) also showed the capsule to be visible only in young cultures of various strains of Streptococci.

The chemical nature of the capsular material varies with different species of bacteria. The capsule of a number of bacterial species consists essentially of a polysaccharide of high molecular weight. This polysaccharide may vary in chemical structure for different strains within a (species). The chemical difference of the capsular carbohydrate in Diplococcus pneumoniae makes for immunological specificity of the different serological types (3). Bovarnick (2) determined the capsule of Bacillus anthracis to be different from a polysaccharide. It was made up of a polypeptide of d(-) glutamic acid.

Chemical nature of dyes used

A dye is defined as an organic compound which contains chromophoric and auxochromic groups attached to benzene rings. The color is attributable to the chromophores and the staining property to the salt-forming auxochromes (6a).

Dyes are spoken of as basic and acidic but these terms do not refer to the actual hydrogen-ion concentration of the dye. Generally, the basic dyes are sold as salts of a colorless acid, and the acid dyes are sold as their sodium, potassium, calcium, or ammonium salts (6b).

Dyes are classified according to their similarity in chemical structure. Crystal violet (6b) is classed as one of the phenyl-methyl dyes. This is a basic dye which has the chemical name of hexa-methyl pararosanilin. Acid fuchsin (6c) is classed as a phenyl methane dye also. This dye owes its acid character to the fact that it is a sulfonated ($-SO_3H$) derivative of basic fuchsin.

Staining Mechanism

Many theories concerning the staining mechanism involved for bacterial systems has been propounded. Two theories which attempt an explanation of staining phenomena are presented here. The first theory is concerned with physical factors. It has been called the "adsorption theory" (10). According to this theory staining results when the dye is deposited and retained on surfaces as a result of physical forces.

The second theory is based on chemical factors (10).

The proponents of this theory state that the color ion of the dye supposedly forms a salt with some reactive group of the stained material. These theories have many followers, but both are somewhat inadequate for the thorough explanation of the staining phenomena.

Generalizations concerning the staining mechanism in technics which involve several steps and the use of mordants are very difficult to derive and support. Other factors must be considered, for the chemical and physical theories are inadequate as they stand now. One of the big factors, recognized by Tolstoouhov (20), which affects the outcome of a staining process is the pH. Using a mixture of acid plus basic dyes at various levels of the staining solution an approximate method for determining the isoelectric point of different strains of bacteria. He showed also that staining with acid dyes is more effective at low pH levels, whereas the basic dyes are most effective within the alkaline range. McCalla (14) also demonstrated quantitatively that the absorption of crystal violet, a basic dye, was greatest on the alkaline side of a reaction, and the reverse was true for the acid fuchsin. Other factors pertaining to the staining process have been observed, but they are incoherent to this study.

EXPERIMENTAL

CHAPTER II

Materials and Methods

Strains of *Streptococcus hemolyticus*, *Streptococcus liquifaciens*, *Klebsiella pneumoniae*, *Klebsiella rhinoschleroma*, and *Escherichia coli* were chosen for study. These were selected from the culture stock in the Bacteriology Department, Montana State University. The organisms had been kept on artificial media for approximately one year. Three strains of the Pneumococcus Type VIII[1,2,3], and one Type IV were obtained from J. A. Hernandez. Before the start of experimentation the morphological and cultural characteristics of each organism, with the exception of the pneumococci, were checked and found to be accurate.

The medium most satisfactory for cultivating the organisms was 1 ml. of sterile beef serum placed on a 10 ml. nutrient agar slant. The pH of the nutrient agar was determined to be 7.1 to 7.2 and the pH of the beef serum varied from 8.3 to 8.5.

The organisms were transferred four successive times at 12 hour intervals before use. The preliminary procedure was employed because the capsule became more evident. Young encapsulated organisms were used. In the first experiments the organisms were incubated at 37° C., but since the results proved inconsistent at times, a temperature of 34° C. was tried and found to be more satisfactory. The relative humidity was held constant by placing a small pan of water in the bottom of the incubator. This latter procedure was found to be of value in enhancing encapsulation.

Two stock staining solutions were prepared; a saturated alcoholic solution of crystal violet, and a saturated alcoholic solution of acid fuchsin. The crystal violet solution was prepared by dissolving 15 g. of dye (90% dye content) in 100 ml. ethyl alcohol. The acid fuchsin was prepared by dissolving 7 g. of dye (80% dye content) in 100 ml. of ethyl alcohol.

The pH of the saturated alcoholic solutions of crystal violet and acid fuchsin was determined electrometrically with a Beckman pH meter, the 5 ml. portions of each dye were individually altered in pH, using .1N HCL, .1N HCL and .1N NaOH as the acid and basic solutions respectively for lowering and raising the pH levels. Samples of the crystal violet solution were adjusted to varying pH ranges of 10.0 to 2.0 using .1N NaOH to raise the pH and .1N HCL to lower it. The acid fuchsin was altered through a pH range of 7.0 to 2.0 using the base and acid as above. .1N HCL was used to lower the pH of both dyes from 2.0 to 1.0. The altered sample of each of the respective dye solutions was placed in a clean pyrex test tube and the tubes were stoppered. It was noted that it would be unfeasible to use a higher pH for both dye solutions. The crystal violet solution at a pH above 10.0 precipitated out in fine particles. Above a pH of 7.3 the acid fuchsin changed color generally but the dye was unsatisfactory as a stain.

Slides used were cleaned in potassium dichromate cleaning solution, and rinsed repeatedly in distilled water.

A non-encapsulated strain of _Escherichia_ _coli_ was used as a control organism.

The Hiss staining technic was used as a control method. The method of Hiss was employed as a standard to gage the effect of the methods under study.

Prior to the commencement of any staining procedure the pH of each solution was checked. This was done twelve hours before making any stained preparations. The cultures were checked for contamination at all times.

Staining Procedures

For the sake of clarity in correlating the staining methods with the results, the procedures are listed in four parts. The first two parts deal with procedures using the various pH solutions of the aniline and alcoholic crystal violet solutions. Part III and IV deal with the use of the various solutions of acid fuchsin.

Part I.

The organisms used were incubated at 37° C. for

18 to 24 hours. As indicated, the staining solutions used in this part were the varying pH samples of the saturated alcoholic crystal violet. A 3 mm. loopful of serum from a tube containing the organisms was placed on a clean slide. The loop was flamed. A loopful of staining solution was spooned out and placed on the drop of serum. The mixture was allowed to stand on the slide for approximately 30 seconds and then mixed in a horizontal rotary motion with the loop so that an equal distribution of stain, about the size of a quarter, was obtained. It was noted that cross-hatching, and zig-zag motions on the stain made for a poor preparation. Stains were allowed to dry and observed around the periphery of the smear for the encapsulated organisms. This procedure was repeated for all organisms with the various solutions of stain. The experiment was repeated twice.

Part II.

The staining solutions used in this part were the varying pH samples of the saturated alcoholic crystal violet. The procedure involved in this part was modified to stain the organism dry, and wash the stain off with 20% copper sulfate. A 3 mm. loopful of serum from a tube containing the respective species of organism was placed on a clean slide. The drop of serum was spread on the slide in a rotary motion, with the loop, to about the size of a quarter. The smear was allowed to dry without heat fixation. The slide was flooded with some of the sample of the solution of the crystal violet and allowed to stand 1 minute. The stain was washed off with 20% copper sulfate. It was then air dried and observed under oil immersion. As in part I this procedure was repeated for all organisms used with the various solutions of the saturated crystal violet. The experiment was repeated twice.

Part III.

The organisms used were incubated at 34° C. for 18 to 24 hours. In this part the procedure was identical with that used in part I except that the various solutions of the alcoholic acid fuchsin were used.

Part IV.

The organisms employed in this part were also incubated at 34° C. for 18 to 24 hours. The procedure used in part II was employed. The various pH solutions of the acid fuchsin were used to stain the smear.

RESULTS

CHAPTER III

Table I indicates the results of the experimental procedure in Part I.

In this series of experiments the capsule appeared negatively stained by the Hiss technic, which was used as a control. The capsule of all organisms used in the procedure of Part I appeared in relief with the use of the saturated alcoholic crystal violet at various pH solutions. However, the capsules possessed by Klebsiella pneumoniae, and Klebsiella rhinoscleromatis were more distinctly stained by the method described than they were by the Hiss technic. Under the conditions of Part I this staining procedure, on the whole, was not as reliable a stain as the Hiss method as regards the other encapsulated species used.

Table II, shows the results of the procedure used in Part II. The capsule, in all encapsulated organisms used, took on a positive stain. The bacterial cell took a deep purple stain and the capsule appeared as a light blue halo surrounded by a distinct stained zone. As compared with the Hiss method this technic was found to be superior.

The results of the procedure used in Part III are recorded on Table III. It was observed that [illegible] sin solutions make poor capsular staining preparations at almost any pH level. Even the bacterial cell of all species appeared hazy between a pH range of 2.5 to 7.0.

In Table IV are recorded the overall results of Part IV.

Table I. Showing results of Part I.

ORGANISM	HISS STAIN	pH 10.0	pH 9.7	pH 9.4	pH 9.0	pH 8.7	pH 8.5	pH 8.3	pH 8.1	pH 7.7	pH 7.5	pH 7.0	pH 6.3	pH 5.6	pH 5.3
Strep. hemolyticus	+++ N	−	−	+ N	++ N	+ N	+ N	+ N	−	−	−	+ N	−	+ N	+ N
Strep. liquefaciens	+++ N	−	−	+ N	++ N	−	−	−	−	−	−	−	−	+ N	+ N
Pneu. IV	+++ N	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pneu. VIII^1	+++ N	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pneu. VIII^2	+++ N	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Pneu. VIII^3	+++ N	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Klebsiella pneumoniae	+++ N	−	−	−	−	−	−	−	−	−	−	+ N	+ N	+ N	+ N
Klebsiella rhino.	+++ N	−	−	−	−	−	−	−	−	−	−	−	−	+ N	+ N
Escherichia coli	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Legend:
− -- capsule not stained
+ -- capsule indistinctly stained
++ -- fair capsule stain
+++ -- good capsule stain
++++ -- very distinct capsule stain

N -- negative capsule stain
P -- positive capsule stain

Table I. Concluded.

ORGANISM	pH 5.0	pH 4.7	pH 4.5	pH 4.1	pH 3.9	pH 3.6	pH 3.3	pH 3.0	pH 2.7	pH 2.4	pH 2.2	pH 2.0	pH 1.7	pH 1.4	pH 1.0
Strep. hemolyticus	+ N	+ N	+ N	+ N	++ N	−	−	−	+ N	+ N	−	+ N	−	−	+ N
Strep. liquefaciens	−	−	−	−	−	−	−	−	−	−	−	−	+ N	+ N	−
Pneu. IV	+ N	+ N	−	−	−	−	++ N	−	−	−	−	−	−	−	−
Pneu. VIII[1]	−	−	−	−	−	−	−	−	−	++ N	++ N	−	+ N	−	−
Pneu. VIII[2]	−	−	−	−	−	−	−	−	−	++ N	++ N	−	−	−	−
Pneu. VIII[3]	−	−	−	−	−	−	−	−	−	++ N	−	−	−	−	−
Klebsiella pneumoniae	+ N	+ N	+ N	+ N	++ N	++ N	+ N	++ N	++ N	++ N	++ N	+++ N	+ N	+ N	−
Klebsiella rhino.	+ N	−	−	+ N	+ N	+ N	+ N	+ N	++ N	+++ N	+++ N	+ N	+ N	+ N	−
Escherichia coli	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Table II. Showing results of Part II.

ORGANISM	HISS STAIN	pH 10.0	pH 9.7	pH 9.4	pH 9.0	pH 8.7	pH 8.5	pH 8.3	pH 8.1	pH 7.7	pH 7.5	pH 7.0	pH 6.3	pH 5.6	pH 5.3
Strep. hemolyticus	+++ N	—	—	++ N	+ N	—	—	—	—	—	—	—	—	—	—
Strep. liquefaciens	+++ N	—	+ N	++ N	+++ N	++ N	++ N	+ N	+ N	+ N	+ N	—	—	—	—
Pneu. IV	+++ N	—	—	—	—	—	—	—	—	—	—	+ N	—	—	—
Pneu. VIII^1	+++ N	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Pneu. VIII^2	+++ N	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Pneu. VIII^3	+++ N	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Klebsiella pneumoniae	+++ N	—	—	—	—	+ N	+ N	—	—	—	—	—	+ N	+ N	+ N
Klebsiella rhino.	+++ N	—	—	—	—	+ N	+ N	+ N	++ N	+ N	+ N	+ N	+ N	+ N	+ N
Escherichia coli	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Legend:
— -- capsule not stained
+ -- capsule indistinctly stained
++ -- fair capsule stain
+++ -- good capsule stain
++++ -- very distinct capsule stain
N -- negative capsule stain
P -- positive capsule stain

Table II. Concluded

ORGANISM	pH 5.0	pH 4.7	pH 4.5	pH 4.1	pH 3.9	pH 3.6	pH 3.3	pH 3.0	pH 2.7	pH 2.4	pH 2.2	pH 2.0	pH 1.7	pH 1.4	pH 1.0
Strep. hemolyticus	−	−	−	−	−	−	+ N	++ P	++ P	++ P	+ N	+ N	+++ P	++ P	+ N
Strep. liquefaciens	−	−	−	++ N	++ N	+ N	+ N	−	−	++ P	++ P	++ P	+++ P	+++ P	+ N
Pneu. IV	−	−	−	++ N	++ N	++ N	++ N	++ N	++ N	++ N	++ N	+ N	+++ P	++ N	+ N
Pneu. VIII[1]	−	−	−	++ N	++ P	+++ P	++ P	++ P	++ P	++ P	++ P	+++ P	+++ P	++ N	+ N
Pneu. VIII[2]	−	−	−	+ N	+ N	++ P	++ P	++ P	++ P	++ P	++ P	+++ P	+++ P	++ N	+ N
Pneu. VIII[3]	−	−	−	++ N	++ N	++ P	++ P	++ P	++ P	++ P	++ P	+++ P	+++ P	++ N	+ N
Klebsiella pneumoniae	+ N	+ N	+ N	++ N	++ N	++ P	++ P	++ P	++ P	++ P	++ P	+++ P	++ N	+ N	+ N
Klebsiella rhino.	+ N	++ N	++ N	++ N	+++ P	++ P	++ P	++ P	++ P	++ P	++ P	+++ P	+++ P	++ N	+ N
Escherichia coli	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Table III. Showing the results for Test III.

ORGANISM	HISS STAIN	pH 7.8	pH 7.6	pH 6.9	pH 6.7	pH 6.5	pH 6.2	pH 6.0	pH 5.7	pH 5.4	pH 5.0	pH 4.7	pH 4.3
Strep. hemolyticus	++ N	—	—	—	—	—	—	—	—	—	—	—	—
Strep. liquefaciens	++ N	—	—	—	—	—	—	—	—	—	—	—	—
Pneu. IV	+++ N	—	—	—	—	—	—	—	—	—	—	—	—
Pneu. VIII^{1}	+++ N	—	—	—	—	—	—	—	—	—	—	—	—
Pneu. VIII^{2}	+++ N	—	—	—	—	—	—	—	—	—	—	—	—
Pneu. VIII^{3}	+++ N	—	—	—	—	—	—	—	—	—	—	—	—
Klebsiella pneumoniae	+++ N	—	—	—	—	—	—	—	—	—	—	—	—
Klebsiella rhino.	+++ N	—	—	—	—	—	—	—	—	—	—	—	—
Escherichia coli	—	—	—	—	—	—	—	—	—	—	—	—	—

Legend:
— -- capsule not stained
+ -- capsule indistinctly stained
++ -- fair capsule stain
+++ -- good capsule stain
++++ -- very distinct capsule stain
N -- negative capsule stain
P -- positive capsule stain

Table III. Concluded

ORGANISM	pH 3.8	pH 3.5	pH 3.3	pH 3.1	pH 2.9	pH 2.7	pH 2.5	pH 2.3	pH 2.1	pH 2.0	pH 1.8	pH 1.4	pH 1.0
Strep. hemolyticus	–	–	–	–	–	–	–	–	–	–	–	–	–
Strep. liquefaciens	–	–	–	–	–	–	–	–	–	–	–	–	–
Pneu. IV	–	–	–	–	–	–	–	–	–	–	–	–	–
Pneu. VIII^1	–	–	–	–	–	–	–	–	–	–	–	–	2+
Pneu. VIII^2	–	–	–	–	–	–	–	–	–	–	–	2+	–
Pneu. VIII^3	–	–	–	–	–	–	–	–	–	–	–	–	–
Klebsiella pneumoniae	–	–	–	–	–	–	–	–	–	–	–	–	–
Klebsiella rhino.	–	–	–	–	–	–	–	–	–	–	–	–	–
Escherichia coli	–	–	–	–	–	–	–	–	–	–	–	–	–

Table IV. Showing the results for Test IV.

ORGANISM	HISS STAIN	pH 7.8	pH 7.6	pH 6.9	pH 6.7	pH 6.5	pH 6.2	pH 6.0	pH 5.7	pH 5.4	pH 5.0	pH 4.7	pH 4.3
Strep. hemolyticus	++ N	—	—	—	—	—	—	—	—	—	—	—	—
Strep. liquefaciens	++ N	—	—	—	—	—	—	—	—	—	—	—	—
Pneu. IV	+++ N	—	—	—	—	—	—	—	—	—	—	—	—
Pneu. VIII^1	+++ N	—	—	—	—	—	—	—	—	—	—	—	—
Pneu. VIII^2	+++ N	—	—	—	—	—	—	—	—	—	—	—	—
Pneu. VIII^3	+++ N	—	—	—	—	—	—	—	—	—	—	—	—
Klebsiella pneumoniae	+++ N	—	—	—	—	—	—	—	—	—	—	—	—
Klebsiella rhino.	+++ N	—	—	—	—	—	—	—	—	—	—	—	—
Escherichia coli	—	—	—	—	—	—	—	—	—	—	—	—	—

Legend:

—	-- capsule not stained
+	-- capsule indistinctly stained
++	-- fair capsule stain
+++	-- good capsule stain
++++	-- very distinct capsule stain
N	-- negative capsule stain
P	-- positive capsule stain

Table IV. Concluded

ORGANISM	pH 3.8	pH 3.5	pH 3.3	pH 3.1	pH 2.9	pH 2.7	pH 2.5	pH 2.3	pH 2.1	pH 2.0	pH 1.8	pH 1.4	pH 1.0
Strep. hemolyticus	−	−	−	−	−	−	−	−	−	−	−	−	−
Strep. liquefaciens	−	−	−	−	−	−	−	−	−	−	−	−	−
Pneu. IV	−	−	−	−	−	−	−	−	−	−	−	−	−
Pneu. VIII^1	−	−	−	−	−	−	−	−	−	−	−	−	+ N
Pneu. VIII^2	−	−	−	−	−	−	−	−	−	−	−	+ N	−
Pneu. VIII^3	−	−	−	−	−	−	−	−	−	−	−	−	−
Klebsiella pneumoniae	−	−	−	−	−	−	−	−	−	−	−	−	−
Klebsiella rhino.	−	−	−	−	−	−	−	−	−	−	−	−	−
Escherichia coli	−	−	−	−	−	−	−	−	−	−	−	−	−

Plate 1.

Klebsiella rhinoscleroma (x3000), as demonstrated at pH 2.0 in procedure of Part II.

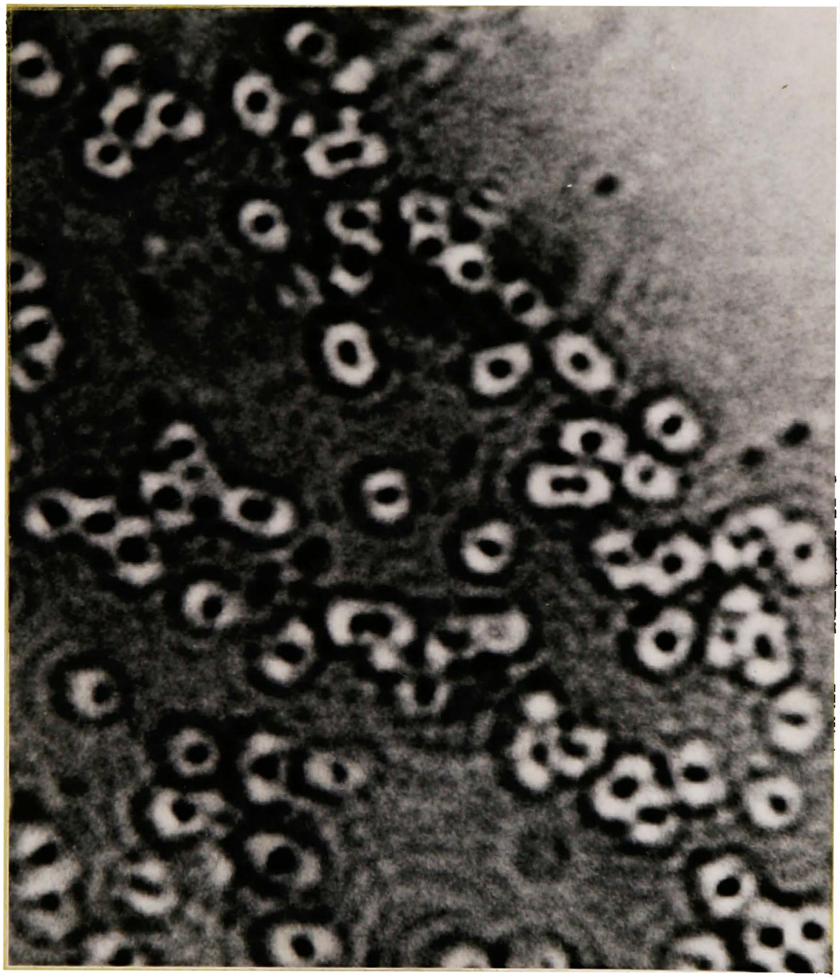

Plate 1

DISCUSSION

CHAPTER IV

A. E. Stearn (16) noted that basic dyes cause an increase in hydrogen ion concentration when these dyes are added to a solution of typical protein. The acid dyes when treated with protein show the effect of decreasing the hydrogen ion concentration. Taking this point into consideration the pH of the dye solutions may have been altered somewhat by the protein in beef serum. Relatively, however the pH of the staining solutions would be constant assuming the effect of the protein in beef serum to be stable.

The capsule stain in Part I had the effect of a relief stain. Klebsiella pneumoniae, and Klebsiella rhinoscleroma at the pH levels of 2.0 and 2.4 displayed well defined capsules. As compared with the Hiss method the capsule observed in these two organisms was easier to demonstrate. The capsule in the other species was stained better by the Hiss method.

The procedure applied in Part II was the most satisfactory one used. The one published procedure, within a limited pH range, displayed a positive staining effect superior to the control method.

It is indicative that the pH values, at which the capsule stained (for most of the organisms used), falls within a low range. No explanation as to why *Streptococcus hemolyticus*, and *Streptococcus liquefaciens* demonstrated a capsule stain within a varied pH range is offered.

Results using the saturated alcoholic acid fuchsin at various pH levels were definitely negative.

One of the initial purposes of these experiments was to note if a dye altered to a certain pH could be used in a practical, simplified and reliable staining procedure. The results are not decisive since one pH solution of the crystal violet or acid fuchsin dye could not be used satisfactorily to stain the capsule of all organisms.

Altering the pH of a saturated basic dye (crystal violet) does have an effect on the capacity of that dye to stain a capsule in the presence of serum. The pH at which the dye stains the capsule most effectively varies with the different organisms. The acid dye (acid fuchsin) was ineffective as a capsular staining material.

SUMMARY AND CONCLUSIONS

CHAPTER V

1. The effect of altering pH on a saturated basic and acid dye for use in a modified bacterial capsule staining technic was studied.

2. Two procedures for staining the capsule were advanced. In one method, the encapsulated organisms were stained as a wet serum preparation. In the second, the stain was flooded on a dry smear, and washed with 20% copper sulfate.

3. Apparently the most satisfactory procedure involved staining a dry smear with the particular pH solutions of crystal violet and washing with 20% copper sulfate.

4. The results using the acid dye (acid fuchsin) were negative.

5. At certain pH levels of the crystal violet dye, the staining method used was superior to the Hiss method.

6. A certain impracticability of the capsule staining procedures was noted. One pH solution of either the crystal violet or acid fuchsin could not be used satisfactorily to stain the capsule of all organisms.

7. It is stressed that more encapsulated organisms, exploring narrow pH ranges could be studied to supplement this indicative work. Also other basic and acid dyes could be used.

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